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MOLECULAR MARKERS FOR PREDICTION OF RESPONSE AND PROGRESSION FREE SURVIVAL TO NOVEL THERAPIES IN CUTANEOUS MALIGNANT MELANOMA

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MOLECULAR MARKERS FOR PREDICTION OF RESPONSE AND PROGRESSION FREE SURVIVAL TO NOVEL THERAPIES IN CUTANEOUS MALIGNANT MELANOMA
THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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In memory of my father, Fernando Antonio de Oliveira Costa.

“Utopia is on the horizon. I move two steps closer; it moves two steps further away. I walk another ten steps and the horizon runs ten steps further away. As much as I may walk, I'll never reach it. So, what's the point of utopia? The point is this: to keep walking”

Eduardo Galeano

POPULÄRVETENSKAPLIG SAMMANFATTNING PÅ SVENSKA

Malignt melanom är en aggressiv tumörsjukdom som har sitt ursprung i huden, slemhinnorna eller ögat. Sjukdomen beter sig delvis olika beroende på var den har sitt ursprung och man handlägger därför dessa typer av melanom på olika sätt. Jag fokuserar på hudmelanom i denna avhandling. Hudmelanom är betydligt vanligare än melanom i slemhinna och ögon. Huvudorsaken till uppkomst av hudmelanom är solexponering, även om andra faktorer som ärftlighet och hudtyp också spelar roll.

Hudmelanom är en av de fem vanligaste tumörformerna och en av de tumörformer som ökar mest i Sverige. Det kan upptäckas in-situ (förstadier) eller när det redan har blivit invasivt. Under 2019 diagnosticerades 4571 invasiva tumörer (hos 4410 individer) i Sverige. Invasivt hudmelanom delas upp i 4 olika stadier beroende på hur mycket tumören har hunnit växa och/eller sprida sig i kroppen. Stadium I och II består av tunna tumörer som bara finns i huden och patienten har betydligt mindre risk för återfall. Stadium III består av hudmelanom som har hunnit sprida sig till lymfkörtlar som ligger nära tumören och stadium IV består av hudmelanom som redan har spridit sig till andra delar av kroppen. Ibland upptäcks dottertumörer (metastaser) av melanom utan att man vet var den primära (ursprungs-) tumören finns någonstans i kroppen.

Behandlingen av hudmelanom anpassas efter stadium I, II, III eller IV av sjukdomen. Stadium I, II och III behandlas huvudsakligen kirurgiskt men man rekommenderar ibland tilläggsbehandling med olika mediciner vid stadium III sjukdom, med syftet att minska risken för att hudmelanom skall komma tillbaka. Denna avhandling fokuserar på patienter som har fått spritt malignt hudmelanom (stadium IV).

Under det senaste decenniet har behandlingen av spritt hudmelanom utvecklats mycket snabbt, vilket har förbättrat överlevnaden avsevärt. Grunden till framstegen baseras på en djupare förståelse av cancerbiologin. Genom att analysera melanomtumörers arvsanlag och proteinuttryck har man sett att ett muterat BRAF protein driver tumörtillväxten i cirka 50% av hudmelanomen, samt identifierat två proteiner som heter CTLA-4 (cytotoxic T-lymphocyte-associated protein 4) och PD-1 (programmed cell death protein 1) (Nobelpris i medicin 2018) som hjälper melanomceller att undgå det egna immunförsvaret.

Patienter med *BRAF* muterat hudmelanom kan nu få behandling med så kallade målriktade behandlingsmediciner som blockerar signalering från muterat BRAF protein och därmed bromsas tumörtillväxten.

Mediciner har även utvecklats mot CTLA-4 och PD-1, vilket leder till att patientens egna immunsystem kan aktiveras och därmed effektivare döda cancerceller.

Dessa behandlingar har inneburit stora framsteg genom att förbättra överlevnaden för många hudmelanom patienter. Tyvärr finns det dock fortfarande många patienter som inte svarar på behandlingen alls och andra patienter som, initialt svarar på behandlingen men som, med tiden

slutar att svara (det vill säga utvecklar resistens). Denna period kan variera mellan några månader och många år (det senare gäller framför allt immunterapi). Vi har idag begränsad kunskap om vilka patienter som kommer att svara bra på behandling och vilka som inte kommer ha någon nytta av behandlingen.

Vi har därför genomfört en studie som består av 4 delar och vars huvudsyfte var att identifiera markörer i blodet eller i tumörer hos patienter med spritt hudmelanom, för att försöka förstå vilken behandling som är bäst för olika patienter (individualiserad behandling). För att kunna göra det behöver vi få en bättre kunskap om vilka markörer som bidrar till uppkomst av resistens mot målriktad behandling och mot behandling som blockerar CTLA4 och PD-1.

Del I

Syftet med studien var att förstå mekanismerna bakom primär- och sekundärresistens till målriktad behandling. Vi jämförde behandlingskänsliga melanomceller med behandlingsresistenta melanomceller och tumörer från patienter som först svarat på behandling med tumörer när de inte längre svarade på behandlingen. Vi hittade proteiner som skiljde sig åt mellan känsliga och resistenta tumörceller och tumörer och såg även att man kunde återskapa känslighet mot behandling i melanomceller om vissa av dessa proteiner blockerades.

Del II

Cancerceller (men även normala celler) frisätter små vesiklar i blodet (så kallade extracellulära vesiklar, EV) när sjukdomen utvecklas och under behandling. EV innehåller bland annat mikroRNA (miRNA) som är en kort RNA som kan reglera uttryck av specifika gener och proteiner. Syftet med denna studie var att identifiera om miRNA i EV kan förutsäga effekten av målriktad behandling. Vi analyserade blod taget före behandlingsstart från 28 patienter varav 25 av dessa patienter även lämnade blodprov ett par veckor efter behandlingsstart. Vi kunde visa att ett högre uttryck av två EV miRNA (let-7g-5p och miRNA 497-5p) under behandling var förenat med bättre behandlingseffekt. Resultatet måste valideras i en större grupp av patienter men visar att EV miRNA sannolikt har betydelse för prediktion av behandlingseffekt vid målriktad behandling.

Del III

Syftet med denna studie var att utvärdera om proteiner i blodet kan identifiera patienter som har större sannolikhet att svara på immunterapi och/eller målriktad behandling. Blodprov från 109 patienter, tagna före-, under och efter behanlding (efter tumörprogress) analyserades. Vi kunde identifiera att 43 kandidatproteiner i plasma, antingen före eller under behandling, associerades till mer långvarig effekt av behandling. Huvudresultatet av studien var att vi kunde dela upp patienterna i två grupper utifrån proteinprofil. En grupp med sämre utfall, med höga inflammatoriska proteinnivåer och låga nivåer av apolipoproteiner, och en grupp med bättre utfall, som hade det omvända; låga inflammatoriska proteinnivåer och höga nivåer av apolipoproteiner. Vissa av dessa proteiner analyserades även i prover tagna när patienter hade fått återfall och vi kunde se en konsistent ökning eller minskning av vissa proteinkandidater

jämfört med prover tagna vid återfall jämfört med innan och under behandling. Studien behöver valideras i en större kohort.

Del IV

Vi tog biopsier (en liten bit) av 28 melanomtumörer innan behandling med målriktad behandling eller immunterapi och analyserade tumörernas genuttryck och korrelerade resultaten med behandlingseffekt. Vi kunde visa att gener relaterade till ett bättre immunförsvar var förenade med bättre svar på målriktad behandling samt att gener relaterade till cellproliferation (tillväxt) var förenade med sämre svar på immunterapi. Våra resultat bekräftade tidigare publicerade studier.

Sammanfattningsvis har studierna i avhandlingen visat att man får viktig information från molekylära analyser av patienternas blodprover och tumörer tagna innan och under behandling, samt när sjukdomen inte längre svarar på behandling. Potentiella prediktiva markörer för behandlingsutfall hos patienter med spritt hudmelanom som behandlas med målriktad behandling och immunterapi har identifierats, men vidare studier i större material krävs för att validera fynden. Studierna belyser värdet av systematisk insamling av biologiska prover tagna före behandlingsstart samt under sjukdomsförloppet för att kunna identifiera nya biomarkörer och även för framtida validering av tentativa prediktiva markörer.

ABSTRACT

Drugs inhibiting the MAPK-pathway (MAPKi) and immune checkpoint inhibitors (ICI) have changed the clinical outcome of metastatic cutaneous malignant melanoma (CMM) in a significant way. Nonetheless, many patients have primary resistance or develop acquired resistance to these therapies within a relatively short period of time. This thesis was performed to explore mechanisms of resistance and possible predictive biomarkers to further improve treatment outcome and to help individualize treatment in this patient population.

In **Paper I**, we compared mRNA and protein expression in MAPKi resistant and sensitive melanoma cell lines. By applying gene expression and proteome profiling we identified two previously described (MET and EPHA2) and two novel (FLI1 and CD13/ANPEP) candidate biomarkers that, when overexpressed, were associated with treatment resistance to MAPKi. The overexpression of MET and EPHA2 was confirmed in melanoma samples from patients with metastatic CMM when comparing samples taken before and after treatment with MAPKi. In cell lines, we demonstrated that an inhibitor of EPHA2 (the multikinase inhibitor dasatinib), re-sensitized cells to MAPKi treatment.

In **Paper II**, we analyzed plasma samples from 28 patients with metastatic CMM before and during treatment with MAPKi. Micro-RNA (miRNA) was extracted from plasmatic extra cellular microvesicles (EVs) and miRNA profiling was performed by microarray, using a panel with 372 human miRNAs. We assessed the association of the miRNA levels with response to treatment and progression free survival (PFS) and found that an increased level of let-7g-5p during treatment, compared to before treatment, was correlated with better response. A high level of miRNA 497-5p during treatment was associated with longer PFS.

In **Paper III**, we investigated if plasmatic proteins were related to response and PFS to MAPKi or ICI in 109 patients with metastatic CMM. Proteomic profiling of plasma samples collected before and during treatment was performed by mass spectroscopy and the abundance of proteins was then correlated with treatment response and PFS. We identified that the plasma levels of 43 proteins, either before or during treatment, were prognostic/predictive of treatment outcome. An inverse correlation between acute-phase inflammatory proteins and apolipoproteins was observed. Patients with high levels of acute-phase inflammatory proteins and low levels of apolipoproteins had worse outcome to therapy.

In **Paper IV**, we analyzed mRNA expression by targeted RNA sequencing of pre-treatment tumor samples from 28 patients with metastatic CMM who underwent treatment with MAPKi or ICI. Transcriptomic data was correlated with treatment response and PFS in gene set enrichment analysis (GSEA). Enrichment of genes in IFN-gamma and inflammatory response was associated with longer PFS to MAPKi therapy, and decreased expression of proliferative genes and increased expression of immune genes correlated with longer PFS to ICI. Finally, lower expression of proliferation genes and immune evasion genes was associated with increased response to ICI.

In summary, we have identified possible mechanisms of resistance and potential predictive biomarkers to novel therapies in patients with metastatic CMM. Our studies were performed in small cohorts of patients and further studies to validate our findings are warranted.

LIST OF SCIENTIFIC PAPERS

I. **Silencing FLI or targeting CD13/ANPEP lead to dephosphorylation of EPHA2, a mediator of BRAF inhibitor resistance, and induce growth arrest or apoptosis in melanoma cells**
Alireza Azimi, Rainer Tuominen, **Fernanda Costa Svedman**, Stefano Caramuta, Maria Pernemalm, Marianne Frostvik Stolt, Lena Kanter, Pedram Kharaziha, Janne Lehtiö, Carolina Hertzman Johansson, Veronica Höiom, Johan Hansson, Suzanne Egyhazi Brage
Cell Death Dis 8, e3029 (2017)

II. **Extracellular microvesicle microRNAs as predictive biomarkers for targeted therapy in metastastic cutaneous malignant melanoma**
Fernanda Costa Svedman, Warangkana Lohcharoenkal, Matteo Bottai, Suzanne Egyhazi Brage, Enikö Sonkoly, Johan Hansson, Andor Pivarcsi, Hanna Eriksson
PLoS One 13, e0206942 (2018)

III. **Inflammation and apolipoproteins as potential biomarkers for stratification of cutaneous melanoma patients for immunotherapy and targeted therapy**
Max J. Karlsson, **Fernanda Costa Svedman**, Abdellah Tebani, David Kotol, Veronica Höiom, Linn Fagerberg, Fredrik Edfors, Mathias Uhlén, Suzanne Egyházi Brage, Gianluca Maddalo
Accepted in Cancer Research (2021)

IV. **Gene enrichment signatures in proliferation and immune response as potential biomarkers for clinical outcome to immune checkpoint inhibitors and to targeted therapy in metastatic cutaneous malignant melanoma**
Fernanda Costa Svedman, Ishani Das, Rainer Tuominen, Eva Darai Ramqvist, Johan Hansson, Veronica Höiom, Suzanne Egyhazi Brage
Manuscript

CONTENTS

LIST OF ABBREVIATIONS

Akt	protein kinase B
AJCC	American Joint Committee on Cancer
ANPEP	aminopeptidase N
APC	antigen presenting cell
APO	apolipoprotein
ARAF	serine/threonine-protein kinase A-Raf
BRAF	v-Raf murine sarcoma viral oncogene homolog B
BRAF	serine/threonine-protein kinase B-Raf
BRAFi	BRAF inhibitor
cfDNA	circulating free DNA
CI	confidence interval
CMM	cutaneous malignant melanoma
CRAF	serine/threonine-protein kinase C-Raf
CRP	C reactive protein
ctDNA	circulating tumor DNA
CTLA4	cytotoxic T-lymphocyte-associated protein 4
DC	dendritic cell
DC	disease control
EPHA2	ephrin type-A receptor 2
ERK	extracellular signal-regulated kinase
EV	extra cellular microvesicle
FDR	false detection rate
FLI1	friend leukemia integration 1 transcription factor
GEP	gene expression signature
Gp100	glycoprotein 100

GSEA	gene set enrichment analysis
HGF	hepatocyte growth factor
HR	hazard ratio
hTERT	h telomerase reverse transcriptase
ICI	immune checkpoint inhibitor
IFN-alfa	interferon alfa
IFN-gamma	interferon gamma
IGFR	insulin growth factor receptor
IHC	immunohistochemistry
IL	interleukin
IRF1	interferon regulatory factor 1
ITH	intra-tumoral heterogeneity
JAK	janus kinase
LBP	lipopolysaccharide binding protein
LDH	lactate dehydrogenase
MAPK	mitogen-activated protein kinase
MAPKi	MAPK inhibitor
MEK	mitogen-activated protein kinase kinase
MEKi	MEK inhibitor
MET	mesenchymal epithelial transition factor receptor
MHC	major histocompatibility complex
miRNA	micro-RNA
mRNA	messenger-RNA
MSigDB	Molecular Signature Database
NF1	neurofibromin 1
NGS	next generation sequencing
NR	non-responder

NRAS	neuroblastoma RAS viral [v-ras] oncogene homolog
OR	odds ratio
ORC1	origin recognition complex subunit 1
OS	overall survival
PD-1	programmed cell death protein 1
PD-L1	programmed cell death 1 ligand 1
PD-L2	programmed cell death 1 ligand 2
PDGFR	platelet-derived growth factor receptor
pERK	phosphorylated ERK
PFS	progression free survival
PI3K	phosphatidylinositol 3-kinase
pMEK	phosphorylated MEK
PTEN	phosphatase and tensin homolog
RAF	rapidly accelerated fibrosarcoma protein kinase
RAS	proto-oncogene protein P21
RECIST	Response Evaluation Criteria in Solid Tumors
ROC	receiver operating characteristic
RR	response rate
SAA	serum amyloid A
SCC	squamous cell carcinoma
SLD	sum of lesions diameter
STAT1	signal transducer and activator of transcription 1
TCR	T cell receptor
TMB	tumor mutational burden
TME	tumor microenvironment
TT	targeted therapy
VEGFR	vascular endothelial growth factor receptor

WES	whole exome sequencing

1 BACKGROUND

Cutaneous malignant melanoma (CMM) has an increasing incidence in both men and women and is the fifth most common cancer type in Sweden, representing approximately 6% of all cancer diagnoses[1]. CMM has a good prognosis when diagnosed in early stages. However, in advanced stages the prognosis is poor even if much progress has been made in the last decade[2]. In Sweden, the mortality is approximately 500 cases per year[1].

A deeper understanding of tumor biology has enabled the development of targeted therapies (TT) and immune checkpoint inhibitors (ICI), that have substantially improved the clinical outcome for patients with inoperable locally advanced and metastatic CMM over the last 10 years. Advanced melanoma has gone from being one of the diagnosis with the most dismal prognosis, with few therapeutic options, to a diagnosis where we have hope to even cure some patients with metastatic disease. However, many patients still have no benefit from these therapies or have benefit only for a limited time due to development of resistance. The discovery of biomarkers predicting outcome and mechanisms of resistance are therefore of utmost importance and still an unmet need, as we strive to improve and personalize treatments for patients.

1.1 THE MITOGEN-ACTIVATED PROTEIN KINASE (MAPK) PATHWAY IN CMM

The MAPK pathway (RAS/RAF/MEK/ERK pathway) is a signal transduction pathway between the cell membrane and the nucleus. Under normal conditions, RAS is activated when different ligands (growth factors, cytokines, hormones) bind to tyrosine kinase receptors on the cell surface[3]. Activated RAS binds to BRAF (and A-RAF and C-RAF) leading to a downstream phosphorylation cascade (pMEK, pERK), culminating in cell proliferation and in cell survival signals inside the nucleus[3] (Figure 1). The *BRAF* gene codes for a serine/threonine kinase protein and was first described as an oncogene in 2002, when somatic mutations were detected in several cancers with a particularly high frequency in CMM (above 50%)[4]. The vast majority of *BRAF* mutations in CMM lead to a substitution of the amino acid valine for glutamic acid at codon 600 (V600E), although other V600 mutations (*i.e.* V600K) may also occur[4-5]. *BRAF* mutations in the kinase domain lead to a constitutive, ligand independent, MAPK pathway activation[4] (Figure 1).

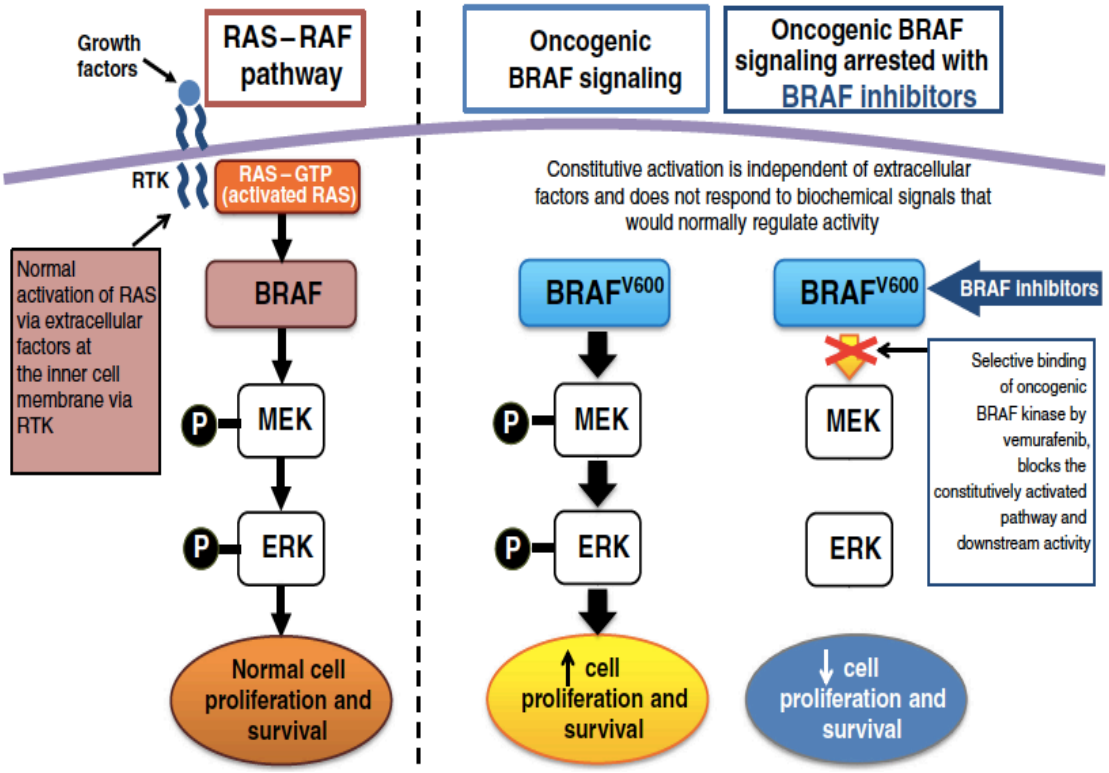

Figure extracted from Ascierto PA, et al. J Transl Med 2012, 10:85.

Figure 1: Illustration of the MAPK pathway activation. To the left, an illustration of the MAPK pathway under normal conditions: MAPK activated by the binding of ligands on cell surface receptors leading to RAS – BRAF activation and phosphorylation of MEK and ERK in the cytoplasm. To the right, an illustration of the effects of an activating *BRAF* mutation and the intervention with a BRAF inhibitor (BRAFi): MAPK is constitutively activated due to the *BRAF* V600 mutation with downstream phosphorylation of ERK, which triggers cell proliferation and cell survival. BRAFi treatments block the constitutively active pathway and can thus reduce proliferation and survival in the cells with *BRAF* mutation[5].

A genomic classification of CMM has been proposed, based on whole-exome sequencing (WES) analysis of more than 300 primary and metastatic CMM. The four proposed subtypes are: *BRAF* (52%), *RAS* (28%,), *NF1* (14%) and triple wild type. *BRAF* and *NRAS* mutations are mutually exclusive so there is minimal overlap. In each subtype, the MAPK pathway is frequently activated[6].

There is also emerging evidence indicating an additional role of the MAPK pathway in oncogenesis related to tumoral immune modulation and immune evasion and that blocking the MAPK pathway can potentially revert this mechanism[7]. For instance, it has been described that melanoma cell lines harboring *BRAF* V600 mutations treated with a MAPK inhibitor (MAPKi) produces significantly less immune suppressive factors like for instance interleukin-6 (IL), interleukin-10 (IL) and vascular endothelial growth factor (VEGF), when compared to untreated controls. Additionally, inhibition of dendritic cell (DC) maturation by these immune suppressive factors was observed[8]. BRAFi and MEK inhibitor (MEKi) treatment have also been demonstrated to increase the expression of melanocyte differentiation antigens and, while

BRAFi increased T cell activation, MEKi decreased T cell function[9], which may be of relevance when combining this type of treatment with ICI. A more extensive explanation about the importance of T cells, DC and cytokines in cancer control is described in detail below.

Other mutations in *BRAF* (beyond V600) have also been described (class II and III *BRA*F mutations[10]) but these are not sensitive to currently approved BRAFi and are therefore beyond the scope of this thesis.

1.2 MAPK PATHWAY INHIBITION IN CMM WITH TT

The characterization of *BRAF* mutations and the role of MAPK pathway in CMM enabled the development of MAPKi with drugs targeting BRAF (BRAFi) and MEK (MEKi), which have been studied in multiple clinical trials, both alone and in combination, achieving impressive results in inoperable locally advanced and metastatic CMM[5,11-20].

There are currently three BRAFi (vemurafenib, dabrafenib, encorafenib) and three MEKi (trametinib, cobimetinib, binimetinib), which have been approved for treatment of patients with inoperable locally advanced and metastatic CMM harboring an activating *BRAF* V600 mutation. The clinical trials described below include this patient population only.

The pivotal study for the vemurafenib approval was the BRIM-3study, a phase III, randomized trial including 675 previously untreated CMM patients. Subjects were randomized to either dacarbazine (the standard of care at that time) or vemurafenib. The response rate (RR) was 57% in patients treated with vemurafenib and 9% for patients treated with dacarbazine. Progression free survival (PFS) was approximately 6 months in the vemurafenib arm and less than 2 months in the dacarbazine arm (hazard ratio (HR) of 0.38, 95% confidence interval (CI) 0.32 - 0.46; $p<0{\cdot}0001$)[11,12].

Similarly, in the registration trial for dabrafenib, the randomized phase III BREAK-3 trial, 733 patients were randomly assigned to either dabrafenib or dacarbazine with a reported RR and PFS for dabrafenib vs dacarbazine of 50% versus 6% and approximately 6 months versus 2 months (HR 0.30, 95% CI 0.18 – 0.51; $p<0{\cdot}0001$)[13,14].

However, although significant improvements in RR and PFS was achieved with BRAFi, about 50% of the patients did not respond to treatment (primary resistance) and 25% of the patients had only short duration (less than 6 months) of response, due to acquired resistance[11-14].

Studies investigating mechanisms of resistance to BRAFi, showed that reactivation of MAPK pathway occurred in the vast majority of the cases, which raised the hypothesis that drug combination with MEKi might more potently inhibit this pathway, with more durable responses[15,16].

The activity of monotherapy MEKi in *BRAF* V600 mutant CMM was also assessed in the randomized phase III METRIC study, where trametinib versus chemotherapy as first line

treatment in 322 CMM patients was compared. The RR was 22% vs 8% and PFS was 4.8 months vs 1.5 months in the trametinib vs the chemotherapy arm (HR 0.45; 95% CI 0.33 - 0.63; p<0.001)[17], which supported the original approval of trametinib monotherapy in CMM.

Thereafter, in at least four phase III randomized trials, treatment naïve CMM patients were included to assess the value of BRAFi plus MEKi combination vs BRAFi alone, and they consistently showed superiority for the combination arm[18,19,21].

In the coBRIM study, 495 subjects were randomly assigned to vemurafenib plus cobimetinib or vemurafenib plus placebo. The RR was better for the combination arm (68% versus 45%, p<0.001). Likewise, the median PFS was 9.9 months versus 6.2 months favoring the combination arm (HR 0.51, 95% CI 0.39 - 0.68; p<0.001)[18]. Additionally, the Combi-D trial included 423 patients that received either dabrafenib plus trametinib or dabrafenib plus placebo. The RR was 69% and 53% in the combination arm and in the dabrafenib arm, respectively (p=0.0014). The median PFS was 11 months in the combination group compared to 8.8 months in the dabrafenib plus placebo group (HR 0.67, 95% CI 0.53 - 0 84; p=0·0004)[19]. The Combi-V study assigned 704 patients to either dabrafenib plus trametinib or vemurafenib alone. The RR was 64% versus 51% (P<0.001) and the median PFS was 11.4 months versus 7.3 months, both favoring the combination treatment (HR 0.56; 95% CI 0.46 - 0.69; p<0.001)[20].

Finally, the Columbus study randomized 577 patients in three arms, encorafenib plus binimetinib, encorafenib alone or vemurafenib alone. The RR was 63%, 51% and 40%, respectively. The median PFS was 14.9 months in the combination arm, 9.6 months in the encorafenib arm (HR 0.75, 95% CI 0.56–1.00; p=0·051), and 7.3 months in the vemurafenib arm (HR 0.54, 95% CI 0.41-0.71; p<0·0001). The reduction in the risk for progression or death with encorafenib alone compared to vemurafenib alone was also statistically significant (HR 0.68, 95% CI 0.52 - 0.90; p=0·007)[21].

In summary, combining BRAFi and MEKi to treat patients with inoperable locally advanced and metastatic CMM with *BRAF* mutation has improved the RR from less than 10% with chemotherapy to almost 70% and the PFS from approximately 2 months to almost 1 year. However, primary resistance exists and acquired resistance occurs in the majority of patients within 1 year after treatment start. Therefore, there is an unmet need to better understand mechanisms of resistance and to unravel biomarkers predicting resistance, which would be informative to guide the development of new therapeutic approaches to overcome resistance and, further improve treatment outcome for our patients.

1.3 THE IMPORTANCE OF THE IMMUNE SYSTEM IN CMM (AND IN OTHER CANCERS)

The transformation of a normal cell into a cancer cell generally requires accumulation of many somatic mutations in the DNA, caused by both intrinsic and environmental factors[22]. CMM is known to have one of the highest numbers of DNA somatic mutations, when compared to many other tumors, which is mainly caused by the skin being exposed to the ultraviolet irradiation of sunlight[23] (Figure 2).

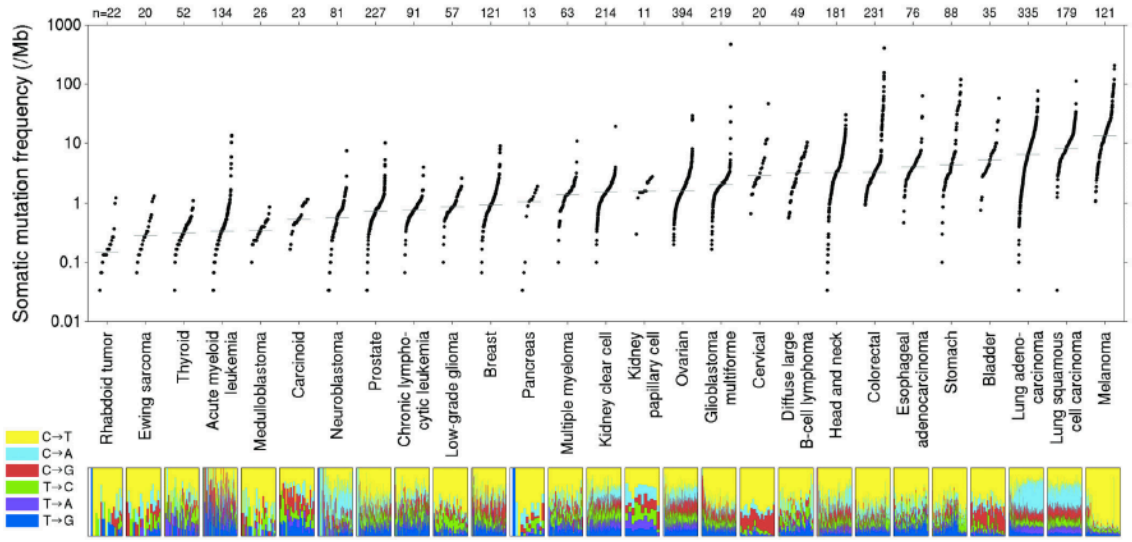

Figure extracted from Lawrence MS, et al. Nature 2013, 499(7457):214-218.

Figure 2: Number of somatic mutations per Megabase in different cancer diagnosis, with CMM on top of the list[23].

Some of these mutations will result in production of tumor specific peptides (neoantigens) that can potentially be recognized by T cells, which have the ability to destroy cancer cells[24]. Many steps in this process, which has been called "The Cancer-immunity Cycle", are necessary to occur in order for T cells to annihilate tumor cells[24] (Figure 3). In brief, it is crucial that the mutations occur in DNA sequences coding for proteins, and that tumor specific altered proteins are presented to T cells by antigen presenting cells (APC), usually DCs. The APC present neoantigens on major histocompatibility complexes (MHC) to T cell receptors (TCR) on T cells. Activated T cells then migrate and infiltrate tumors recognizing the neoantigens on MHC molecules presented on cancer cell surface and hopefully kill tumor cells. Tumor cell death leads to further release of neoantigens and restarts and strengthens the cancer-immunity cycle[24].

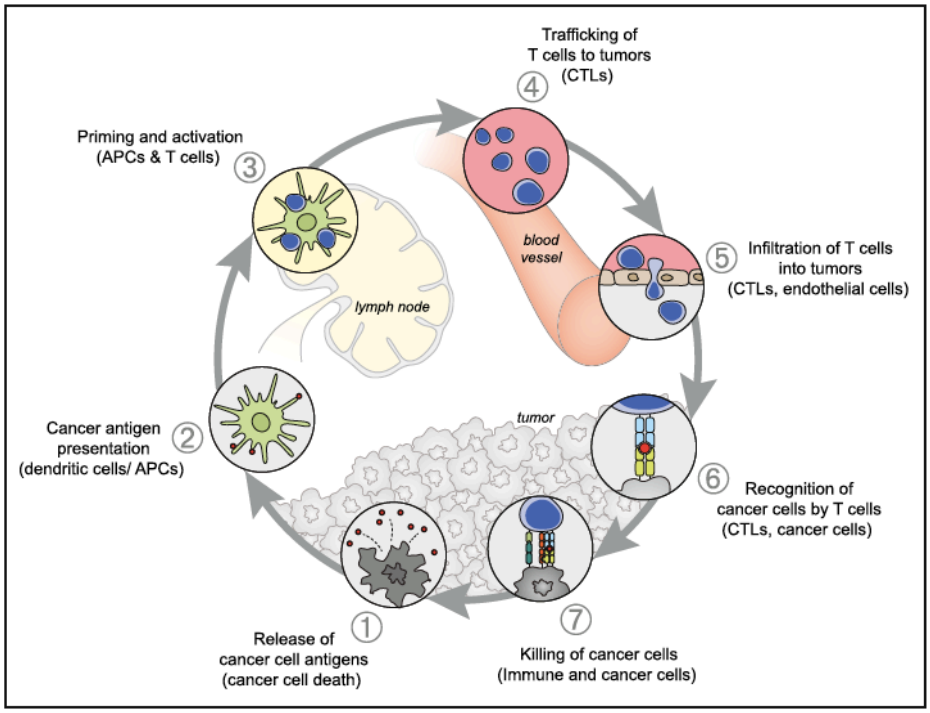

Figure extracted from Chen DS, Mellman I. Immunity 2013, 39(1):1-10.

Figure 3: The seven steps in the Cancer-Immunity Cycle, from neoantigens release by tumor cells, recognition and presentation to T cells by APC/DC. T cells circulate and infiltrate tumors, finally recognizing and killing tumor cells[24].

However, this process is extremely complex and requires the interplay of many stimulatory and inhibitory molecules, as well as the release of many cytokines in each step, in order to properly destroy tumor cells effectively without causing auto immunity[24,25]. Many of these inhibitory molecules and their ligands are presented in Figure 4.

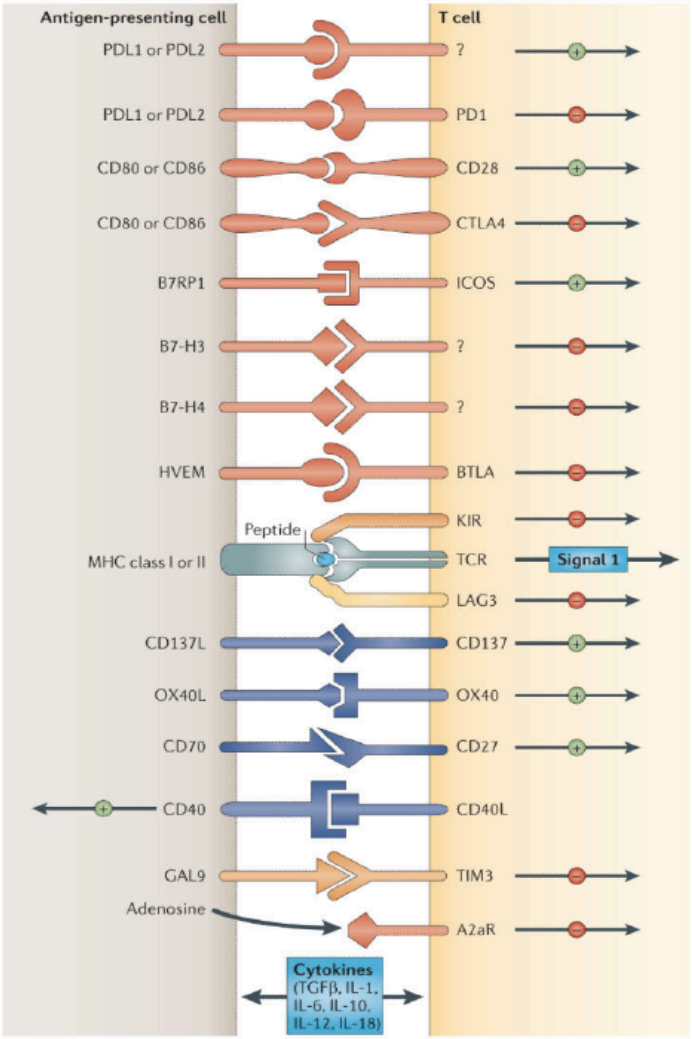

Figure extracted from Pardoll DM. Review Nat Rev Cancer 2012, 12(4):252-264.

Figure 4: The complex interaction between stimulatory (in blue) and inhibitory (in red) molecules expressed in APC and in T cells, after the antigen (peptide) presentation by APC through major MHC to TCR. The secretion of many cytokines by T cells is essential for these processes to happen[25].

The most well studied inhibitory proteins are the immune checkpoints cytotoxic T lymphocyte-associated protein 4 (CTLA-4) and the programmed cell death protein 1 (PD-1), which have been documented as immune evasion mechanisms by cancer[25,26] (Figure 5). ICI targeting the proteins CTLA-4 and PD-1, or its ligand PD-L1, have been developed and are approved for multiple tumor types, including renal cell carcinoma, lung cancer, head and neck cancer, urothelial cancers, and many others[27].

After antigen presentation to T cells by APC, co-stimulatory ligands (CD 80 or CD86) from APC bind to its stimulatory receptor CD28 on T cells and thereby, activating them. However, the same ligands may bind even more strongly to CTLA-4 on inactivated T cells and thereby, inhibit T cell activation[24,26]. One of the key players in this process is IL-2 secreted by newly activated T cells, leading to T cell proliferation and survival[26]. A similar process that occurs

later on in the cancer immunity cycle is observed inside the tumors, to where activated and primed (first time activated) T cells migrate, recognize and kill tumors cells. The inhibitory PD-1 signaling pathway may become activated by the ligation of PD-1 ligands 1 and 2 (PD-L1 and PD-L2) produced by tumor and/or stromal cells to the immune inhibitory receptor PD-1, which is expressed on T cells. This interaction impairs T cell proliferation and survival. Interferon-gamma (IFN-gamma) produced by the activated T cells is a central regulatory factor in this adaptive process, as explained below[24,26].

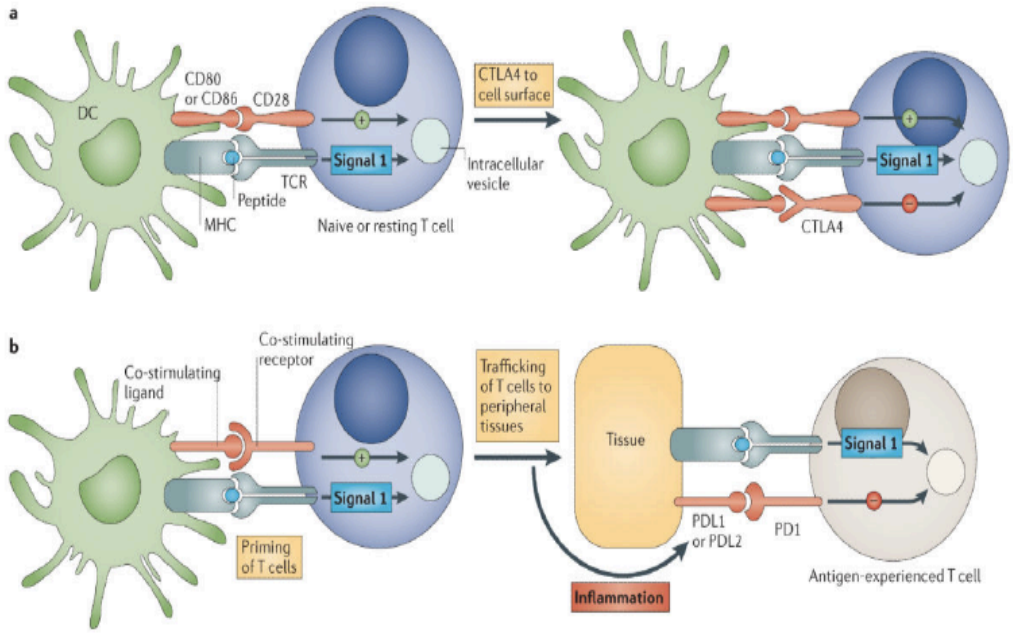

Figure extracted from Pardoll DM. Review Nat Rev Cancer 2012, 12(4):252-264.

Figure 5: Illustration of the mechanisms of the two main CTLA-4 and PD-1 immune checkpoints. CTLA-4 functions as a brake on the signal at the time of the T cells initial response to antigen (a). PD-1 functions as a "brake" primarily by regulating the inflammatory/immune response by activated T cells, recognizing antigen in peripheral tissue (b)[25]. Exploiting this knowledge, antibodies blocking CTLA-4 and PD-1/PDL-1 (checkpoint inhibitors) have been developed and quickly implemented in the clinic during the last decade. Anti-PD1 has dramatically improved clinical outcome for patients with inoperable locally advanced and metastatic CMM, as it is described below.

1.4 CHECKPOINT INHIBITION IN CMM

There are currently three approved ICI in Europe to treat patients with inoperable locally advanced and metastatic CMM, ipilimumab (anti-CTLA-4), nivolumab and pembrolizumab (anti-PD-1).

The first ICI approved was ipilimumab, and the pivotal phase III study randomized 676 previously treated CMM patients in three treatment arms (ipilimumab plus glycoprotein 100

melanoma antigen peptide vaccine (gp100 vaccine), ipilimumab alone or gp100 vaccine alone). Although the median PFS was the same in the 3 arms (less than 3 months), the median overall survival (OS) was 10 months in both groups receiving ipilimumab versus 6.4 months in gp100 vaccine alone (HR 0.6; p=0.003). The RR was less than 6% for ipilimumab plus gp100 vaccine, 10.9% for ipilimumab alone (p=0.04) and 1.5% for gp100 vaccine alone[28]. The results encouraged further trials with ipilimumab against CMM as first line treatment.

A first line phase III trial with ipilimumab was then performed randomizing 502 patients to either ipilimumab plus chemotherapy (dacarbazine), or to dacarbazine plus placebo. The difference in RR was small, with 15% in the ipilimumab plus dacarbazine arm and 10 % in the dacarbazine arm (p=0.09). However, the duration of response was 19 months versus 8 months favoring the group that received the combination (p=0.03)[29].

The anti-PD-1 drug nivolumab was approved few years later. Relevant studies for this approval were the randomized phase III clinical trials CheckMate 037 and CheckMate 066[30,31]. The first study included previously treated patients with ipilimumab, and also with BRAFi in cases with a *BRAF* mutation, whereas the second study was performed in previously untreated CMM patients. In the CheckMate 037 trial patients were treated with either nivolumab (n=272) or chemotherapy (n= 133) (dacarbazine or carboplatin). This patient population was heavily pre-treated and a higher proportion of subjects with increased levels of lactate dehydrogenase (LDH) and brain metastasis (both known as negative prognostic factors) was observed in the nivolumab arm. Still, the RR was 27% versus 10% for nivolumab and chemotherapy, respectively. However, the median PFS was similar for both groups (3.1 months for nivolumab and 3.7 months for chemotherapy (HR 1.03, 95.1% CI, 0.78 - 1.436), as was the median OS (16 months versus 14 months) (HR 0.95, 95.54% CI 0.73 - 1.24). Nevertheless, a significant number of patients in the chemotherapy arm received ICI as subsequent treatment, which make the OS analysis difficult to interpret. The authors observed longer duration of responses with nivolumab compared to chemotherapy (32 months v 13 months), concluding that nivolumab was a treatment option for this patient population[32].

In the CheckMate 066 study, 418 CMM patients were randomized to nivolumab or dacarbazine as first line therapy. They observed a RR of 40% vs 14% (odds ratio (OR), 4.06; p<0.001), and a median PFS of 5.1 months vs 2.2 months with nivolumab and dacarbazine, respectively (HR 0.43, 95% CI 0.34 - 0.56; p<0.001)[31].

The main trials for the approval of pembrolizumab were the randomized phase II and III trials, Keynote-002 and Keynote-006[33,34]. The Keynote-002 study randomly assigned 540 heavily pre-treated patients to either 2 different doses of pembrolizumab (2 mg/kg versus 10 mg/kg every 3 weeks), or to the chemotherapy of choice by the investigator. Patients had previously received ipilimumab and those with *BRAF* mutations had also been treated with MAPKi. The RR and PFS were not significantly different between the two pembrolizumab dose levels. The RR was above 20% in the pembrolizumab arms and only 4% in the chemotherapy arm (p<0.0001). The PFS was reported as the restricted mean duration based on 12 months of

follow-up and was around 5.5 months for pembrolizumab and 3.6 months for chemotherapy, HR of (HR 0·57, 95% CI 0·45–0·73; p<0·0001)[35].

In the phase III Keynote-006 study, 834 patients were randomized into three arms consisting of two different dosing schedules for pembrolizumab (10 mg/kg every 2 or 3 weeks) versus ipilimumab. Both pembrolizumab arms showed the same efficacy, which was superior to the ipilimumab arm. The RR was around 33% and 12% for the pembrolizumab arms and for the ipilimumab arm, respectively (p<0.001), and the median PFS was approximately 5 months for the pembrolizumab arms and hardly 3 months for the ipilimumab arm (HR 0.61; 95% CI 0.50 – 0.75; p<0·0001)[34,36].

Although these studies have showed that ICI is a considerable breakthrough in the treatment of CMM with some long-lasting responses, the majority of patients do not respond or do not present durable responses with any of these treatments alone. Studies evaluating the effect of the combination of ICI were thus warranted. The first phase III combination trial reported was CheckMate-067. This clinical trial randomized 945 patients into three treatment arms: nivolumab plus ipilimumab, ipilimumab alone or nivolumab alone. Importantly, the study was not powered to compare the nivolumab+ipilimumab arm with nivolumab alone. The RR was 58%, 45% and 19% for the combination, nivolumab monotherapy and ipilimumab monotherapy, respectively. The median PFS was 11.5 months (95% CI 8.7-19.3) in the combination arm, 2.9 months (95% CI 2.8-3.2) in the ipilimumab arm, and 6.9 months (95% CI 5.1-10.2) in the nivolumab arm. However, the observed high toxicity of the combination was a major limitation, with 59% of the patients in the combination arm developing grade 3 and 4 side effects, in comparison with only 23% and 28% grade 3 and 4 side effects with nivolumab alone and ipilimumab alone, respectively[37]. OS strongly supports the value of using combination treatment. This trial reported the highest 5-year OS for patients with locally advanced and metastatic CMM so far, 52% with the combination compared to 44% with nivolumab alone and 26% with ipilimumab alone[37].

It should be noted that the best treatment modality (MAPKi or ICI) as first choice to a CMM patient population harboring an activating *BRAF* mutation is not established, and data from clinical trials comparing different sequencing strategies are still not mature[38,39]. Results from phase III trials combining MAPKi and ICI have not shown robust improvement in PFS so far[40,41].

1.5 MOLECULAR BIOMARKERS PREDICTING TREATMENT OUTCOME IN CMM

MAPKi and ICI has changed the treatment paradigm for patients with advanced CMM, but we still do not fully understand how to identify the patients who will benefit from treatment. Therefore, discovery and validation of reliable biomarkers that consistently predict mechanisms of primary and acquired resistance to therapies is an unmet need. Identifying such biomarkers may help to select optimal first line treatment for patients- some may benefit from

monotherapy, others from combinations or different sequences of treatments. Improved patient selection may save costs, decrease unnecessary side effects, and may potentially contribute to increased treatment access as many novel drugs are expensive. Identifying resistance mechanisms may also help to identify new drug targets and may provide a rationale for new treatment combinations.

Prognostic biomarkers are associated with clinical outcome regardless of treatment, while biomarkers that are predictive of treatment benefit anticipate if a patient will benefit from a specific treatment or not. Some biomarkers can be both prognostic and predictive and, in some cases, biomarkers may be druggable[42].

In CMM there is a body of evidence supporting the relevance of various biomarkers. However, there is currently only one biomarker with a clear cut-off value (present or not) that can safely enough preclude that a specific patient will not benefit from a specific approved treatment, which is *BRAF* wild type status in relation to BRAFi therapy[43]. The aim of this section is to provide a description of key selected publications in the field of research on prognostic and predictive biomarkers in advanced CMM.

1.5.1 Molecular biomarkers predicting treatment outcome to currently approved MAPKi in CMM

As mentioned, the unique predictive biomarker currently available for BRAFi treatment is the presence of activating *BRAF* V600 mutations. Tumors with wild type *BRAF* will not have benefit from BRAFi treatment. Nevertheless, we still can not identify the subgroup of patients with primary resistance to MAPKi, even if they have a positive *BRAF* mutation status, and we cannot foresee which patients will experience durable responses[4,43].

Baseline clinical and pathological characteristics were compared with treatment outcome to BRAFi alone or BRAFi+MEKi, in two pooled larger retrospective cohorts of treatment naïve patients for advanced disease[44,45]. The smaller study (n=142) included subjects on BRAFi monotherapy or BRAFi+MEKi and found female sex, normal baseline LDH level, *BRAF*V600E genotype, as well as response to treatment, independently associated with better outcome to MAPKi[44]. The larger study (n=563) comprised only patients treated with the combination. Factors reflecting high tumor burden (high baseline LDH level, three or more of organs compromised by metastasis and the sum of lesions diameter (SLD) of 66 mm or more) were most significantly associated with worse outcome. However, almost 60% of the patients without these poor features (*i.e*. normal LDH, less than 3 compromised organs and SLD of less than 66 mm) developed progressive disease within 3 years[45]. These clinical features are probably more linked with prognosis than with prediction.

Although substantial research has been conducted exploring mechanisms of resistance to MAPKi in *melanoma tissue*, no useful biomarker beyond *BRAF* assessment has been generated to be used in clinical praxis. However, studies revealing that MAPK reactivation is the most

common mechanism of resistance to BRAFi monotherapy contributed to improved treatment options, by suggesting that a combination of BRAFi + MEKi would improve the outcome[16,18,19,21,46]. The most commonly identified alterations involved in MAPK pathway reactivation and PI3K-pathway activation (another commonly activated pathway in resistant lesions) are *NRAS* mutations, *BRAF* amplification, *MEK* mutations, *NF1* mutations, *Akt* amplification, loss of *PTEN,* and increased expression of growth factors and of cell membrane receptors, such as hepatocyte growth factor (HGF), tyrosine-protein kinase MET receptor, platelet derived growth factor receptor (PDGFR), insulin growth factor receptor (IGFR)[47] as illustrated in Figure 6.

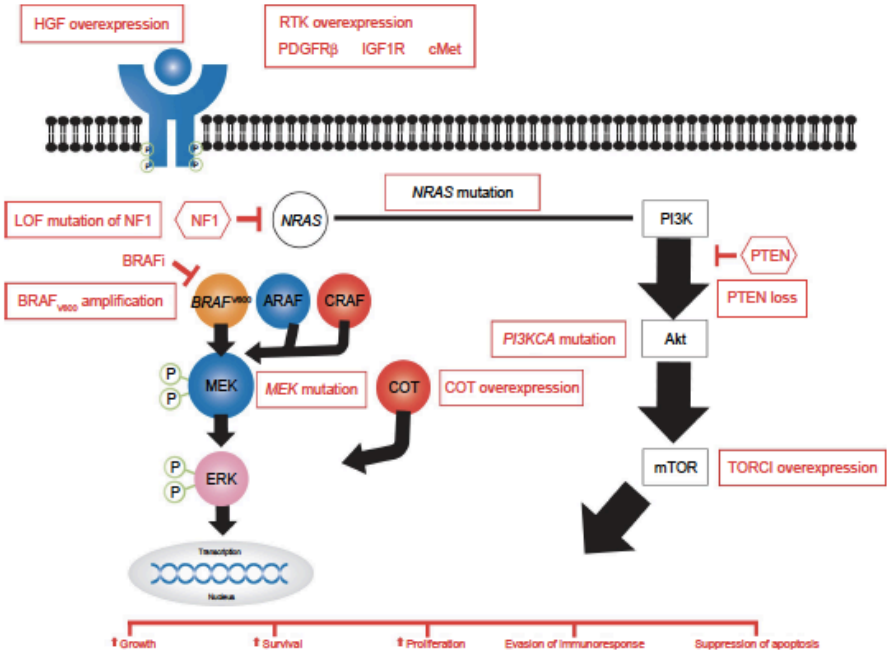

Figure extracted from Kakadia S, et al. Onco Targets Ther 2018, 11:7095-7107.

Figure 6 illustrates some different mechanisms of resistance to MAPKi leading to reactivation of oncogenic pathways MAPK and PI3K[47].

As for the role of MEKi in *BRAF* wild type tumors, some data indicate that at least a subset of patients likely have benefit from MEKi treatment in the *BRAF* wild type population[48]. Importantly, there are multiple new MAPKi in development and also new BRAFi in development that target classes II and III (non V600) *BRAF* mutations, where currently approved BRAFi have no efficacy[49,50]. Most likely, there is thus a future for MAPKi also in other subsets of patients than those within the *BRAF* V600 mutant segment.

A recently recognized mechanism of resistance to BRAFi/MEKi relates to tumoral immune evasion caused by *BRAF* mutation and increased immune response against cancer cells when impairing MAPK pathway activity[47,51,52]. Interestingly, increased expression of immune genes (*i.e.* IFN-gamma signature) has been correlated with higher MAPKi efficacy[51,52].

Some of the findings above were revealed by studying melanoma tumor biopsies, but the process of identifying tissue biomarkers is complicated by tumor heterogeneity and branched evolution/clonality as the disease progresses[16].

The importance and complexity of tumor heterogeneity is illustrated by Shi H et al, who performed WES analysis in melanoma samples resistant to BRAFi, showing that MAPK pathway reactivation was present in 70% of the cases (mainly due to *RAS, MEK, CDKN2A* mutations, *BRAF* amplification and *BRAF* splicing mutation variants). They also showed that PI3 kinase pathway activation (by *PI3KCA, PI3CG, PI3Kr2, PTEN, PHLPP1* mutations) was the second most important reason to acquired resistance (22% of the tumors). WES of germline DNA, baseline samples and multiple sequential samples acquired over time from some patients, together with an evolutionary clonal analysis, revealed evidence of branched clonality and high levels of intra and inter-tumoral heterogeneity, with sometimes different pathway activation mechanisms[16]. These findings indicate that multiple tumor biopsies from multiple metastases may be necessary over time to detect molecular tissue biomarkers in each individual tumor and patient. Furthermore, at least in the case of MAPKi and CMM, some of the most common mechanisms of resistance should maybe be approached through relevant combination therapies in front line therapy (before an extensive branched clonality is established).

Circulating tumor biomarkers have the advantage of being easily accessible and of providing biological information that may reflect several tumoral cellular subpopulations. The presence of *BRAF* V600 mutation detected in cell-free circulating DNA (cfDNA) in baseline plasma samples was independently correlated with worse clinical results to MAPKi in a large retrospective study. The authors analyzed a pool of over 700 patients previously included in clinical trials with dabrafenib or trametinib[53]. As expected, high tumor burden correlated with the presence of *BRAF* V600 mutation cfDNA in plasma. This observation was also independently correlated with worse RR, PFS and OS to dabrafenib or trametinib in the multivariate analysis, adjusting for known negative prognostic characteristics (performance status, plasmatic LDH levels, sum of the largest lesions and disease staging). All relevant patient subgroups had benefit from dabrafenib or trametinib compared to chemotherapy, which indicate that baseline *BRAF* V600 mutation cfDNA is mainly a negative prognostic biomarker[53].

1.5.2 Molecular biomarkers predicting treatment outcome to currently approved ICI therapies in CMM

Some easily accessible *clinical baseline characteristics* (presence of liver metastasis, increased tumor burden, impaired clinical conditions and symptomatic brain metastasis) and routinely requested *blood samples* (LDH levels, high C-reactive protein (CRP) levels, low lymphocytes and high neutrophile count) have been associated with worse outcome to ICI in melanoma[54]. However, patients with these conditions (in particular impaired clinical condition and symptomatic brain metastasis) are usually excluded from phase III clinical trials, and it is

unclear if these characteristics are negative prognostic or predictive factors. Other clinical information, such as the development of immune-related side effects, increasing circulating lymphocytes and eosinophile counts seem to correlate with better treatment outcome, although it should be pointed out that this is based on retrospective data[54].

Lower levels of *circulating tumor DNA* (ctDNA) has recently been associated with better outcome to ICI as first line therapy in more than 200 melanoma patients. The study also suggested that patients with higher levels of ctDNA may benefit from combined treatment with anti-CTLA-4 and anti-PD-1 instead of monotherapy[55].

Many studies have assessed the importance of *tissue biomarkers* such as tumor mutational burden (TMB), T cell infiltration, PD-L1 expression, gene expression signatures and their association with treatment benefit from ICI. A summary of some of the key papers published in this area is described below.

As previously described, the first step necessary to start the cancer immunity cycle is a proper neoantigen release by tumor cells and its presentation to T cells by APC. Melanomas are characterized by a very high number of genetic mutations compared to other tumors. The level of genetic alterations is also frequently referred to as TMB, and a high TMB results in an increased possibility that immunogenic neo-antigens are presented[23,24]. It is thus not surprising that RR and TMB have shown to be significantly correlated (correlation coefficient of 0.74) in a pooled analysis of patients with different tumor indications treated with ICI. The RR was significantly higher in patients with tumors with high mutational load[56]. In addition, a high clonal (present in all tumor cells) neoantigen burden in tumor samples has been correlated with more homogeneous tumors (regarding neoantigens) and longer OS in lung cancer patients and also in 64 CMM patients receiving ICI. CMM patients with tumors harboring a low neoantigen intra-tumoral heterogeneity (ITH) and a high clonal neoantigen burden had a longer OS. ITH is suggested to generate more subclonal neoantigens (present in only some of the tumor cells), which are not as effective in activating T cells as the clonal antigens[57].

It has been hypothesized that the machinery necessary for antigen presentation, MHC class I and II molecules, could be predictive to response to ICI. MHC class I down-regulation has been shown to be associated with resistance to anti-PD-1 immunotherapy in clinical material consisting of biopsy material collected at baseline and on progression[58]. Further analysis showed that transforming growth factor beta is likely contributing to the development of resistance and down-regulation of MHC class I[58]. Other data indicate that MHC class II may also be relevant. In cell lines, MHC class I was shown to be ubiquitously expressed in 60 different melanoma cell lines while MHC class II was only expressed in half of the cell lines. A gene set enrichment analysis (GSEA) comparing MHC class II positive and negative cells revealed an enrichment of genes involved in immune processes like PD-1 signaling, T cell receptor signaling, graft-versus-host disease and allograft rejection in MHC II + cells[59]. A correlation between increased protein expression of MHC class II by IHC and better clinical results to ICI was demonstrated in two small independent melanoma patient cohorts.

Additionally, high MHC class II expression also correlated with high T cell infiltration (CD4+ and CD8+) in tumors and with high PD-L1 expression in melanoma cell lines[59].

Increased T cell infiltration and TCR clonal expansion in melanoma samples have been shown to be associated with better effect of ICI[54]. In a study using next generation sequencing (NGS) and immunohistochemistry (IHC), including tumor biopsies collected before and during treatment with anti-PD-1, baseline tumors in patients who responded to anti-PD-1 had higher expression of CD8+ T cells in the invasive tumor margin, compared to non-responders. An increased expression of CD8+ T cells in the tumor center was observed when analyzing samples collected during treatment from responders but not in non-responders. Additionally, PD-1 and PD-L-1 expression was significantly increased in the invasive tumor margin and inside the tumors in patients who responded to anti-PD-1, compared with those who had progressive disease during treatment. The authors could predict outcome by using a logistic model correlating CD8+ T cells infiltration in baseline tumor samples and probability of response to anti-PD-1, in a small validation cohort consisting of 15 additional patients. A multiplexed immunofluorescense assay showed that response to treatment and the proximity between PD-1 and PD-L1 was significantly correlated. Response was also correlated with PD-L1 expression in tumor cells and with PD-1 expression in CD8+ T cells. A more diverse repertoire of TCR was detected with NGS analysis using pre-treatment and on treatment DNA in responders compared to non-responders, and also after treatment with anti PD-1[60].

High PD-L1 expression is correlated with better outcome to ICI even in large cohorts of melanoma patients included in pivotal clinical trials, however even patients with negative or unknown PD-L1 status benefit from treatment[31,37]. There are further issues when considering PD-L1 expression as a predictive biomarker i.e., different antibodies assays are available using different cut-offs and scoring systems, some considering PD-L1 expression only in tumor cells, whereas others also take PD-L1 expression in TME cells into consideration[61].

Different signaling pathways also play a central role in tumor immune sensitivity, and the most studied one in this context is the IFN-gamma signaling pathway. In brief, activated T cells in TME secrete IFN-gamma, which binds to its receptor (IFN-gamma R) on the tumor cell surface initiating a downstream cascade by activating janus-kinase (JAK)1 and JAK 2 and signal transducer and activator of transcription 1 (STAT1) in the cellular cytoplasm. This process activates the interferon regulatory factor 1 (IRF1), which is a transcriptional regulator of PD-L1 transcription. PD-L1 protein migrates to the cellular membrane and finally it ligates with PD-1 receptor in T cells, leading to T cell inactivation, as demonstrated in Figure 7[62].

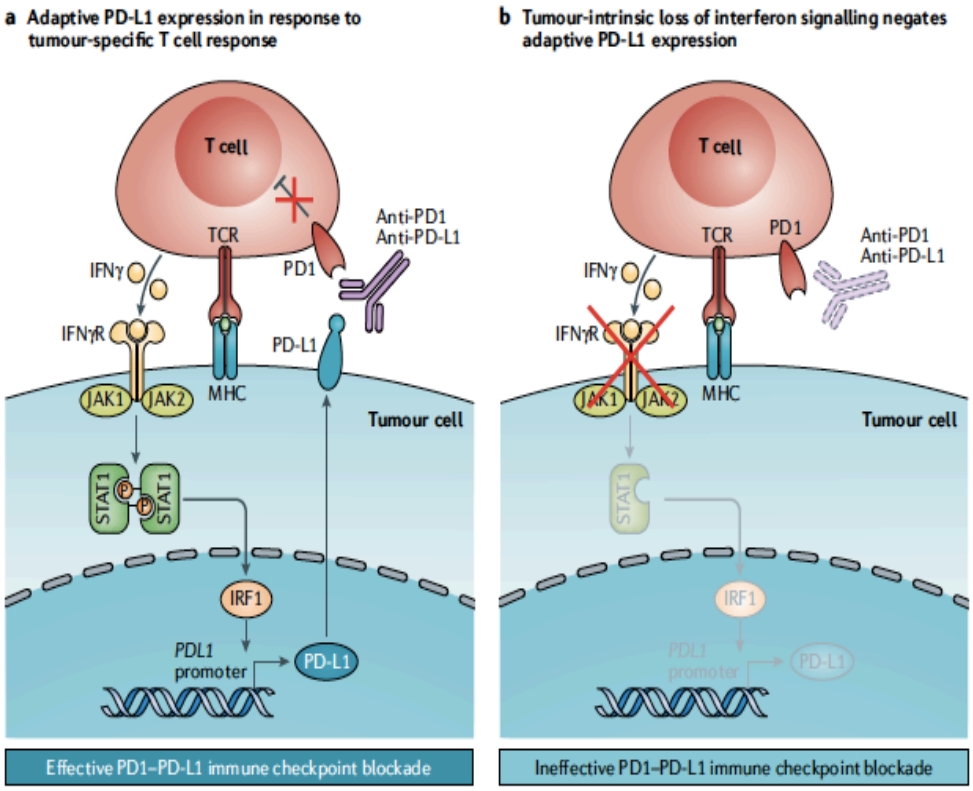

Figure extracted from Kalbasi A, Ribas A. Nat Rev Immunol 2020, 20(1):25-39.

Figure 7. The IFN-gamma signaling pathway as described above, which is the rationale behind the potential predictive role of the IFN-gamma gene signatures[62].

Since the mechanism of action of anti-PD-1 is dependent on PD-1/PD-L1 interaction, it is likely that the IFN-gamma signaling pathway and high expression of its associated genes in melanoma correlate with response to anti-PD-1 treatment[63]. In fact, when comparing top ranked genes differentially expressed between responders and those that did not respond, in a cohort of limited sample size with melanoma patients treated with ICI, a 10 gene signature was generated from a panel of almost 700 tumor and immune genes. This gene signature was baptized as "preliminary IFN-gamma signature". The predictive potential of this signature was confirmed in a similar validation cohort, and the signature was expanded to 28 further immune genes. Further validation testing in other cohorts, including subjects with diverse tumor subtypes (all treated with ICI), finally led to a generation of a T cell inflamed gene expression signature (GEP) (where 6 genes are from the original preliminary IFN-gamma signature), which correlated with both better response and longer PFS[63]. However, up-regulation of immune genes have also shown to be prognostic in CMM and, in fact, up-regulation of immune genes also correlated with better outcome to MAPKi[6, 51,53].

The prediction performance of T cell inflamed GEP and TMB was investigated in more than 300 patients with different tumor origin included in four Keynote (pembrolizumab) clinical trials. Inflamed GEP and TMB showed independent predictive performance and provided orthogonal information. The authors conclude that inflamed GEP and TMB should be used in future clinical trials design[64].

In another study, 266 CMM samples were classified as T cell inflamed or non-T cell inflamed, based on differentially expressed T cell gene signature, and then compared in order to identify differentially active pathways. β-catenin signaling pathway was significantly more active in non-T cell inflamed melanomas compared to T cell inflamed tumors. Expression of β-catenin signaling pathway genes was inversely correlated with CD8A (mirror CD8+ T cell) gene expression in non-inflamed compared with inflamed melanomas. The authors confirmed in mouse models that melanomas with active β-catenin signaling present T cell exclusion and resistance to ICI[65].

The role of activated MAPK pathway and immune modulation in melanoma has already been described in the previous section on MAPKi related biomarkers but it is worth pointing out this information also in the context of ICI therapy[7].

Finally, there is a growing interest and evidence about the relationship between the gut *microbiome*, immunity, efficacy and toxicity of ICI in cancer[66]. Several clinical trials are currently enrolling patients globally to examine the impact of the manipulation of microbiome with diet alteration, probiotics and fecal transplantation on cancer outcome.

2 AIMS

The overall aim of this thesis was to identify new mechanisms of resistance and potential predictive molecular markers for treatment response and PFS to TT/MAPKi and ICI in patients with metastatic CMM.

The specific aims were:

- **Paper I**

To identify differentially expressed mRNA and proteins between BRAFi sensitive parental melanoma cell line and BRAFi resistant daughter cell lines.

To investigate if biomarker candidates associated with BRAFi resistance in cell lines also correlate with BRAFi resistance in patients.

- **Paper II**

To determine whether circulating extra-cellular vesicular microRNAs (EV miRNA) can serve as predictive biomarkers for response and PFS to TT.

- **Paper III**

To investigate if proteins detected in plasma can serve as predictive biomarkers for TT and ICI.

- **Paper IV**

To correlate gene expression profiling in tumor samples with response and PFS to TT and ICI in order to identify predictive biomarker candidates.

3 MATERIAL AND METHODS

A brief general description of material and methods in the different papers is described below. Detailed and more specific information are to be found in each paper.

3.1 PATIENTS

Subjects included in all 4 papers were patients with metastatic CMM classified according to the 7th edition of the American Joint Committee on Cancer (AJCC) Melanoma Staging Database[67] and treated at the Department of Oncology at Karolinska University Hospital, in Stockholm. The majority of cases were treated and followed outside of clinical trials, following our clinical routines, which includes a radiological evaluation every 2-3 months. Patients were classified as responders/disease control (DC) if presenting complete response, partial response or stable disease, and as non-responders (NR) in cases with progressive disease. Response to treatment was based on the Response Evaluation Criteria in Solid Tumors (RECIST) 1.1. However, a strict and formal application of RECIST criteria when evaluating radiological imaging is not routinely conducted in our clinic for patients not participating in clinical trials. PFS was relevant for papers II – IV and was calculated from the date of treatment start until confirmed disease progression, date of death or date of last follow up, whatever came first. Some patients were under treatment with TT or ICI within clinical trials and were followed according to specific routines recommended by the study protocols.

Relevant clinical information for prognosis like sex, age, baseline plasmatic levels of LDH and stage were assembled and correlated with response to treatment and PFS in papers II – IV.

3.1.1 PAPER I

Five patients with stage IV CMM disease (M1c) were included in this study, two females and three males. All of them were treated with TT as first line treatment for metastatic disease (vemurafenib n=3, dabrafenib n=1 and dabrafenib + trametinib n=1). Their age at therapy start ranged between 23 and 66 years. Four of them responded to treatment initially, but acquired resistance over time, and one patient never responded to therapy (primary resistant).

3.1.2 PAPER II

Twenty-eight stage IV CMM patients (16 males and 12 females) treated with BRAFi monotherapy (n=18), or in combination with MEKi (n=10) as first line treatment (except one case who previously received ipilimumab), between March/2012 and May/2015, were included in this study. Age ranged between 32 and 80 years. All of them had metastatic disease (M1c n=26, M1b n=1, M1a n=1) and the majority of cases (n=19) had increased baseline plasmatic LDH levels, ranging between 2.6 and 58.3 microKat/L. Regarding tumor response, 68%

obtained DC and 25% were NR. For 7% of the subjects, response data was not available due to premature death. The PFS ranged between 18 and 627 days (median PFS 177 days).

3.1.3 PAPER III

One-hundred and nine stage IV CMM patients receiving TT (n=44; BRAFi monotherapy n=21 and BRAFi+MEKi n=23) or ICI (n=65; anti-CTLA4 n=10, anti-PD1 n=52 and anti-CTLA4+anti-PD1 n=3) were investigated in this study. The majority of the subjects received first line treatment (95% for TT and 80% for ICI). One patient treated with TT and 4 cases treated with ICI, had received 2 prior lines of therapy. The study participants were treated between December/2011 and August/2017. Twenty-five of 44 and 42 of 65 patients were male in the TT and the ICI cohorts, respectively. The median age was higher in the ICI group compared to the TT group (66 versus 60 years), and the majority of patients in both treatment sets had M1c disease (n=41 of 44 and n=45 of 65). The median plasmatic levels of LDH were 4.6 (range 2.6-58.3 microkat/L) and 4 (range 1.7-26.5) for TT and ICI, respectively. A more complete description of patient characteristics can be found in Table 1 in the manuscript.

3.1.4 PAPER IV

Twenty-eight stage IV CMM patients receiving TT (n=13; BRAFi as monotherapy n=10, BRAFi+MEKi n=3) or ICI (n=15; anti-CTLA4 n=1, anti-PD-1 n=14) between March/2012 and March/2017, participated in this study. Patients were treatment naïve with the exception of one patient, who received ICI as third line therapy, after two prior lines of chemotherapy.

Detailed patient characteristics can be found in Table 1 in the manuscript. In brief, there were more men than women in both cohorts and patients were older in the ICI group, with a median age of 73 compared to 63 years in TT group. Most patients had M1c disease but more patients on ICI therapy had M1a/b disease (6 ICI versus 2 TT). Plasmatic levels of LDH were higher in the TT group compared to ICI group (median 5.3 versus 4.1 microkat/L).

In this manuscript, patients were assessed for not only overall clinical response and PFS but also local tumor response (from the biopsy site, as explained below).

In the validation cohort for origin recognition complex subunit 1 (ORC1) (one of the top candidates) protein expression analysis with IHC, 24 patients (6 of them overlapped with the ICI cohort) were included. These patients had similar characteristics as the cohorts described above, shown in Supplementary Table 19 in the manuscript.

3.2 CELL LINES

3.2.1 PAPER I

A commercially available BRAFi sensitive melanoma cell line (A375), harboring the *BRAF* V600E mutation, and BRAFi resistant daughter cell lines, generated by repeatedly exposing the parental cell line to BRAFi (PLX4720 or vemurafenib), were used in the study. Primary BRAFi resistant melanoma cell line SKMEL24, harboring *BRAF* V600E mutation, was also used for some of the analysis.

3.3 METHODS

3.3.1 ETHICAL CONSIDERATIONS

Our studies were conducted following Good Clinical Practice/the Declaration of Helsinki and ethical approvals were obtained from Stockholm Regional Ethics Committee, Sweden. Informed consent was acquired from included subjects in these four studies.

3.3.2 PAPER I

DNA, RNA and proteins were extracted from BRAFi sensitive A375 and BRAFi resistant daughter cell lines and targeted next generation sequencing (NGS), gene expression and mass spectrometry-based proteome profiling to unravel BRAFi resistance mechanisms were performed. Additionally, qPCR and western blot were used to validate the findings.

A subset of candidate markers identified in *in vitro* models were evaluated by IHC in tumor samples collected from 3 patients before treatment and at disease progression (total 6 samples). The protein expression was scored based on the intensity and on the percentage of melanoma cells stained by 4 independent examinators, blinded for the clinical data. Furthermore, RNA was extracted from fresh frozen melanoma biopsies sampled before treatment and at disease progression from 2 additional patients (in total 4 samples). Targeted RNA sequencing was done using the Ion Ampliseq Transcriptome Human Gene Expression kit (Thermo Fisher Scientific, USA). Quantitative PCR was performed for candidate genes of interest.

3.3.3 PAPER II

Plasma samples were collected from 28 patients before treatment with TT and also from 25 of these patients during treatment. Extracellular vesicles (EVs) were isolated from plasma and miRNA was extracted from the EVs and analyzed by miRNA microarray, using a panel of 372

human miRNA. After data normalization, EV miRNA levels in samples before and during treatment, as well as the difference between EV miRNA levels during treatment compared to before treatment (delta_values), were correlated with response to treatment and to PFS. Moreover, levels of the candidate miRNAs were determined by qPCR in eight patients with matched plasma samples provided before, during treatment and at disease progress. The correlation of clinical characteristics (sex, age and LDH plasmatic levels pre-treatment) with disease outcome (RR and PFS) was also investigated.

3.3.4 PAPER III

The total number of subjects in the study was 109. Plasma samples taken at baseline were available from 98 patients (35 TT and 63 ICI patients), on-treatment samples were available from 85 patients (27 TT and 58 ICI patients) and samples collected at recurrence were available from 30 patients (19 TT and 11 ICI). Plasmatic proteins were diluted and digested to peptides, which were labelled before tandem mass spectroscopy analysis was performed (LC/MS/MS). Sequentially, raw data was normalized, and possible hemolysis and coagulation effects eliminated as described in the manuscript. Relative abundance data for plasmatic proteins were generated (relative because each sample was compared to a pool of 40 randomly selected plasma samples balanced for sex, type of therapy, pre/on-treatment samples). A linear model correlating protein abundance in each sample with PFS was built, adjusting for sex, stage and age. A machine learning model including the identified proteins that were significantly associated with treatment response was developed, and receiver operating characteristic (ROC) curves were generated, to demonstrate the performance of the predictive models.

3.3.5 PAPER IV

RNA was extracted from fresh frozen tumors samples. After controlling for quantity and quality (using the Agilent Bioanalyzer 2100), a targeted transcriptomic sequencing analysis was applied, by using the Ion Ampliseq Transcriptome Human Gene Expression kit, which detects > 20,000 RefSeq genes. Following data normalization, a correlation between the transcriptomics input and therapy outcome (clinical response, tumor specific response and PFS) was investigated. A GSEA with 50 hallmark gene sets from the Molecular Signature Database (MSigDB)[68] was performed to identify gene sets differentiating between responders/DC and NR and association to PFS. Finally, protein expression of ORC1, one of the potential predictive candidate markers for ICI, was examined by IHC in an additional cohort of formalin-fixed paraffin embedded melanoma tumors from the Pathology Archive, Karolinska University Hospital.

4 RESULTS

4.1 PAPER I

We induced resistance by treating A375 melanoma cell line with BRAFi and generated three different BRAFi resistant daughter cell lines, which were also cross resistant to trametinib (MEKi). The proteomic profiling revealed 49 proteins significantly upregulated in all three daughter cell lines compared to the parental cell line. Of these proteins, two were new biomarker candidates for resistance (FLI1 and CD13/ANPEP), and two have previously been described as possible resistance factors (EPHA2 and MET). An increased expression of mRNA of these candidate markers was associated with BRAFi resistance. Importantly, silencing FLI1 or EPHA2 re-sensitized cells to BRAFi, an effect observed also in primary BRAFi resistant SKMEL24 cells. A down-regulation of FLI1 and phosphorylated EPHA2 proteins, and of FLI1 and EPHA2 mRNA, was observed when exposing primary and secondary resistant melanoma cells to the multi-kinase inhibitor dasatinib. Combining dasatinib with vemurafenib reverted both primary and acquired resistance to BRAFi.

The protein expression of MET and EPHA2 was increased, as detected by IHC, in all three melanoma metastases after progression compared to the melanoma tumor biopsies taken at baseline (all three patients initially responded to TT). Regarding the two additional patients contributing with baseline and recurrence melanoma samples for the mRNA expression data, one was a responder and the other a non-responder (NR). The baseline melanoma biopsies were compared and FLI1 and EPHA2 expression were significantly higher in the NR, while ANPEP and MET expression were lower. Further comparison between the baseline samples and the samples collected at disease progression demonstrated that MET was overexpressed in both progression tumors, in line with the protein expression results. FLI1 and EPHA2 mRNA expression increased after progression compared to the pre-treatment sample in the responder, but not in the NR. On the other hand, ANPEP was overexpressed in the progression sample from the NR, compared to baseline expression, but this was not seen in the sample from the responder. In order to validate the Ampliseq results, qPCR was applied. However, the qPCR results were not always in line with the Ampliseq data. qPCR analysis of the tumor from the patient that responded to TT showed down-regulation of FLI1 expression at progression.

Taken together, the most consistent result when observing cell lines and tumor data in our material (protein and mRNA expression) is that increased expression of MET and EPHA2 may be relevant acquired mechanisms of resistance.

4.2 PAPER II

We found that higher levels of EV let-7g-5p during treatment compared to pre-treatment (delta_value), were associated with better response to TT (OR 8568.4, 95% CI = 4.8–1.5e+07,

P = 0.000036) and that elevated levels of EV miRNA 497-5p during therapy correlated with longer PFS (HR = 0.27, 95% CI = 0.13–0.52, P <0.000061). We analyzed matched plasma samples before, during treatment and after disease progression from 8 patients. The two candidate miRNAs were evaluated by qPCR, to investigate if we could see a significant difference in their levels after disease progression compared to pre and during treatment samples. We could not see a significant difference regarding EV let7g-5p levels. EV miRNA 497-5p was not detected in five of eight samples collected during progression, which may suggest that EV miRNA 497-5p is down-regulated at progression. However, EV miRNA 497-5p was also not detected in samples from 3 patients collected during therapy.

Among the clinical characteristics, baseline LDH level was independently associated with PFS in a multivariate analysis adjusting for age and sex (HR = 2.1, 95% CI = 1.03–4.2, P = 0.04). However, no significant correlation was observed between plasmatic LDH with response to treatment.

4.3 PAPER III

The relative abundance of 43 plasmatic proteins, in samples taken either pre- or on-treatment with ICI or TT, were found to be independent (in a model including age, stage and sex) correlated with PFS (as illustrated in Figure 2 in the manuscript). Four proteins had a predictive performance for ICI treatment benefit in the samples taken during treatment: the acute-phase inflammation protein lipopolysaccharide binding protein (LBP) and the apolipoproteins APOC1, APOA4 and APOA1. An inverse correlation between the relative abundance of apolipoproteins and acute-phase inflammation proteins was clearly observed in a clustered heat map for the whole cohort. Patients with longer PFS had lower levels of acute-phase inflammation proteins and higher levels of apolipoproteins. High abundance of apolipoproteins and low abundance of acute-phase inflammation proteins also correlated with better response to treatment regardless of TT or ICI treatment (Figure 3 in the manuscript).

Based on the machine learning generated models, protein abundance levels on-treatment for patients receiving ICI had the best performance.

4.4 PAPER IV

4.4.1 TT cohort

We have correlated age, gender, pre-treatment plasmatic levels of LDH and M stage with response and PFS. None of these clinical features were significantly correlated with response, nevertheless, elevated LDH levels was independently correlated (adjusted for age, gender and

stage) with worse PFS (HR 1.12, 95% CI 1.00 - 1.27, P = 0.04). Patients with DC had longer median PFS (245 days) than those primarily resistant to TT (60 days).

Tumor specific response and overall clinical response was discordant in four patients (clinical response: DC=7 and NR=6; tumor response: DC=11 and NR=2), which may be an effect of tumor heterogeneity.

When applying a p-value of <5% and a false detection rate (FDR) of 20%, 23 genes reached significance for tumor specific response separating DC and NR. One gene (PRKG2) was significant for overall clinical response. Furthermore, a significant association between 5 genes and PFS was detected, among them PSMB8, STAT1 and CD8A.

In GSEA, no signature significantly correlated (FDR<20%) with response, probably due to the very small number of patients. However, enrichment of genes in immune and inflammatory pathways significantly correlated with better PFS, some of them with a FDR of less than 5%. For instance, gene enrichment in hallmarks for inflammatory response, IFN-gamma response, IFN-alfa response and in IL6-JAK-STAT3 signaling were among the hallmarks that were significantly correlated with longer PFS.

4.4.2 ICI cohort

None of the clinical features (age, gender, pre-treatment plasmatic levels of LDH and M stage) correlated with response to ICI but increased LDH levels was marginally correlated with shorter PFS (HR.1.42, 95% CI 0.99-2.05, p=0.058), after adjusting for age, gender and stage. As expected, response to ICI correlated with median PFS (DC=1260 days, NR=55 days).

The clinical response and specific tumor response were discordant in only one patient treated with ICI (clinical response: DC=12 and NR=3; tumor response: DC=10, NR=4). One case was not evaluable for tumor response due to tumor resection before the radiological evaluation.

Six genes were statistically significantly associated with tumor specific response and 7 genes significantly associated with clinical response ($p < 0.05$ and FDR <20%). No gene was significantly associated with PFS (< 6 months, 6 months - 2 years, > 2 years), probably due to the small number of patients in each group.

In GSEA, down-regulation of gene sets involved in proliferation (*i.e.* hallmarks E2F targets, G2M checkpoint, mitotic spindle, MYC targets) and in immune evasion (hallmarks WNT beta catenin signaling) were significantly associated ($p < 0.05$, FDR <20%) with better tumor specific response. A decreased expression of proliferative gene sets (hallmarks E2F targets, G2M checkpoint, MYC targets) and increased expression of immune genes (hallmarks allograft rejection) significantly correlated with longer PFS (< 6 months, 6 months - 2 years, > 2 years).

5 DISCUSSION

5.1 PAPER I

This paper identifies two novel (FLI1 and ANPEP/CD13) and two previously described (EPHA2 and MET) proteins contributing to TT resistance in melanoma, by comparing differentially expressed proteins and mRNA in melanoma cell lines sensitive and resistant to TT. Additionally, a consistent overexpression of MET (both at the protein and the mRNA level) was observed in clinical tumor samples from CMM patients collected after progression compared to baseline samples. Increased expression of EPHA2 was also observed in all samples collected after progression (either by IHC or Ampliseq) from patients who were classified as responders. Furthermore, baseline expression of FLI1 and EPHA2 (mRNA) was significantly elevated in the non-responder compared with the case with disease control, which may indicate their role in primary resistance as well. The mRNA expression of ANPEP in the clinical material was more inconsistent and difficult to interpret. However, this is a small cohort of cases and no strong conclusions can be drawn.

MET is a transmembrane tyrosine kinase receptor normally present in epithelial cells, which in cancer can be activated either by its ligand, the HGF (which is usually secreted by mesenchymal cells), or in a ligand independent manner. Activation leads to cellular proliferation, survival, invasiveness, motility, etc. This happens mostly through the activation of the MAPK pathway, but also by other pathways such as PI3K[69]. MET overexpression has been seen in melanoma, and the role of HGF/MET as a resistance mechanism to TT in melanoma has been demonstrated[69-71]. Straussman R and co-workers showed that melanoma cells are more sensitive to BRAFi when cultivated alone than when co-cultivated with stromal cells. Proteomic analysis demonstrated that HGF secreted from fibroblasts cause activation of MET, which leads to re-activation of the MAPK- and PI3K pathways. This study suggested that HGF/MET was involved in primary resistance and, although pre-clinical experiments combining MEKi+BRAFi reduced the negative effect of HGF/MET, it could not eliminate it. However, inhibiting HGF/MET could re-sensitize cells to BRAFi. Further investigation in clinical melanoma samples confirmed that expression of HGF in tumor stroma and phosphorylated MET correlated with poorer outcome to treatment[72]. Indeed, cabozantinib, a tyrosine-kinase inhibitor against MET and VEGF, has shown efficacy in patients with advanced melanoma, (n=77; uveal, cutaneous and mucosal) in a phase II trial, where 66% of the patients were previously treated in the metastatic setting. Of 42 patients with CMM in the study, 5 patients had partial responses and 29 stable disease on cabozantinib monotherapy[73].

The tyrosine-kinase receptor EPHA2 is reported to promote oncogenesis (besides its physiological functions in *i.e.* embryogenesis) and becomes activated when binding to ephrin, but also when binding to other EPHA2 receptors on the surface of neighboring cells. This generates a special cell-cell activation and may be important in the light of our findings, as the overexpressed receptors can activate each other and lead to increased cell survival,

proliferation, adhesion, migration, etc. The membrane-nucleus signaling is described to occur through different pathways, like MAPK- and PI3K[74]. There is evidence in the literature that EPHA2 is overexpressed in melanoma cells and tissues, and is an important player in melanoma progression, contributing to a more aggressive behavior[75]. Previous data have also shown that EPHA2 expression is correlated with primary and secondary resistance to TT in melanoma cell lines. Besides, it has been demonstrated that it is overexpressed in clinical melanoma samples after tumor progression with TT, in comparison with baseline tissue[76]. Notably, it has been shown that EPHA2 inhibitors may overcome resistance to BRAFi therapy in cell lines[76]. In our paper, we have also demonstrated that it was possible to re-sensitize resistant cells to BRAFi by exposing them to dasatinib, a multi tyrosine kinase inhibitor, which inhibits EPHA2 expression.

FLI is a transcription factor associated with cancer development by both activating and suppressing different genes involved in proliferation, apoptosis and angiogenesis. FLI is known to be overexpressed in many different tumors, including melanoma cell lines and melanoma tumors samples[77,78]. In CMM, FLI-1 expression has been shown to be higher in metastatic tumors compared to primary melanoma, and is significantly correlated with features associated with tumor aggressiveness, such as ulceration and high proliferation, as assessed by Ki-67 expression[78]. As mentioned above, our study is the first to correlate high FLI-1 expression with resistance to TT. This is relevant since some molecules, including commonly used antitumor medicines (*i.e.* etoposides), have been shown to inhibit FLI-1 in tumor cell lines and may easily be explored in combination with MAPKi in the clinic[79,80].

ANPEP/CD13 has been observed on the membrane of melanoma cells (but not on melanocytes), and in experiments using Matrigel, seems to play an important role in cellular adhesion, communication and, invasion[81]. Data in the literature has shown that transfecting melanoma cells with ANPEP/CD13 increase their invasive ability[82]. In our study, we found a down-regulation of phosphorylated EPHA2 when blocking CD13 using an antibody. ANPEP/CD13 should be further investigated as a mechanism of resistance to TT since it may be a potential therapeutical target.

The results from our study and the data in the literature warrant further research combining MAPKi with drugs inhibiting some of these targets (*i.e.* MET, ANPEP/CD13 and EPHA2). This strategy should be considered in not only heavily pre-treated CMM patients, but also in patients with known clinical and pathological features associated with poor prognosis, despite treatment with BRAFi+MEKi[45].

5.2 PAPER II

In this study, we have identified two plasmatic EV miRNAs as possible predictive biomarker candidates for response (let-7g-5p) and longer PFS (miRNA 497-5p) to MAPKi in metastatic CMM patients, by performing miRNA microarray analysis of circulating EV miRNA. Comparing EV miRNA levels in baseline plasma samples between patients with favorable and

unfavorable outcome (response and PFS) did not generate statistically significant different results. However, increased delta-levels of EV let-7g-5p (difference between on treatment and pre-treatment levels) and higher levels of EV miRNA 497-5p during treatment were associated with better response and longer PFS, respectively.

Although our results were generated in a small cohort of subjects without a control group, our findings are plausible. miRNAs regulatory impact on gene expression is often described as repressive at the post-transcriptional level and as described in the discussion part of our paper, RAS has been shown to be down-regulated by let-7 miRNA family members. The miRNA 497-5p has been associated with down-regulation of RAF, MEK and ERK proteins[83-85].

Since the publication of our work some interesting data has been published, mainly regarding EVs and miRNA 497. For instance, Harmati M and coworkers have demonstrated that, after exposing mouse melanoma cells to heat, chemotherapy and hypoxia, an increase in the number of EVs and changes in their cargo (like miRNAs and proteins) could be observed. Besides this, they could show that EVs released by tumor cells influence TME, for instance by increasing mesenchymal stem cells proliferation through upregulation of Ki-67[86].

Luo G et al have recently published an extensive review of the literature about miRNA 497 expression in tumors, non-cancerous tissues, cancer cells lines, and plasma and its correlation with cancer diagnosis, prognosis and sensitivity to cancer treatment. This review emphasizes the correlation between low miRNA 497 expression with cancer diagnosis, worse prognosis and resistance to cancer treatment (indicating a tumor suppressive role for miRNA 497) in different tumor types[87]. In another study, a comparison about miRNA expression between 36 melanoma samples and 36 age-matched benign nevi detected a down-regulation of miRNA 497-5p (and miRNA 195-5p and miRNA 455-3p) in tumor samples and that human telomerase reverse transcriptase (hTERT) mRNA expression was inversely correlated with these miRNAs. A lower expression of these miRNAs correlated with higher cell proliferation, programmed cell death inhibition and a more aggressive malignant behavior in melanoma cell lines[88]. Finally, Mizrahi A at al studied differences in miRNA expression during the evolution from normal skin to the development of squamous cell carcinoma (SCC). They analyzed archived tissue samples of normal skin, solar elastosis, keratinocytic intraepidermal neoplasia and SCC. The miRNA 497 was increasingly down-regulated in each phase of the process from normal skin towards SCC. Further studies on cell lines demonstrated that miRNA 497 reduced cell motility, by changing cell phenotype from a spindle-shaped mesenchymal to an epithelial form, indicating MET involvement. In fact, epithelial-mesenchymal transition mRNA genes were down-regulated in cells over-expressing miRNA 497 compared to control cells[89]. These data are consistent with the findings of our study where high EV miRNA 497 content was a positive prognostic factor.

5.3 PAPER III

We identified 43 proteins as potential prognostic/predictive biomarkers in patients treated with MAPKi and ICI. We could separate the 109 patients in our cohort into two groups, based on the protein levels detected in plasma. One group was characterized by increased levels of acute-phase inflammation proteins and decreased levels of apolipoproteins, while the other group had the opposite pattern. Patients in the prior group had a worse outcome to therapy compared to the latter group. To exclude that acute inflammation was actually caused by high tumor burden, and therefore not adding additional information, we have adjusted the proteomic data by disease stage (AJCC 8th edition)[2], LDH levels and number of metastatic organs. Still, the levels of 15 proteins remained significantly correlated with outcome in the multivariable analysis.

The role of inflammation in cancer has been debated for many years and yet, it was not included in the initial review of hallmarks of cancer of Hanahan and Weinberg in 2000[90]. A growing body of evidence supports the importance of immune escape and role of inflammation in cancer, which led Hanahan and Weinberg to add ”avoiding immune destruction” as a hallmark, and “tumor-promoting inflammation” as an enabling characteristic in cancer development in their 2011 update[91]. Acute activation of the innate immune system is known to play a key role for our survival, by eliminating pathogens, cancer cells and promoting wound healing. However, there is also evidence that a persistently activated innate immune system is oncogenic. A chronic inflammation with consistently elevated local production of cytokines, growth factors and metalloproteases initiate processes involved in cell division, invasion, survival, vascularization, and not least, they impair the function of the adaptive immune system against cancer[92]. Data also support that a long-term deficient adaptive immune system is associated with increased tumor development, which is evident from an increased cancer incidence in immune deficient mouse models, in patients receiving immune suppressive drugs or with certain viral diseases known to attack the adaptive immune system (*i.e.* HIV)[91, 92].

Evidence in the literature for other tumor forms, support our findings concerning acute-phase inflammation proteins detected in plasma being prognostic[93]. In general, acute-phase inflammation proteins are produced in the liver. But it has also been shown that melanomas themselves may produce some acute-phase proteins (SAA2), which can help them to evade the adaptive immune system by increasing immune suppressive neutrophils in the TME[94,95]. Local tumor production of serum amyloid A1 (SAA1) has also been reported in glioblastoma[96].

Apolipoproteins have been described to be down-regulated during inflammation and high levels have been shown to be protective against cancer, whereas low levels have been associated with increased risk of developing cancer[97-102]. Low levels have also been reported to be prognostically unfavorable in multiple indications[103-106]. There is data supporting that apolipoproteins may be involved in a range of functions that relate to cancer development, as well as resistance to therapies. Interestingly, in the context of the work presented in this thesis, preclinical data indicate that adding apolipoprotein A reduces proliferation and increases apoptosis through down-regulatedregulation of MAPK signaling, and also that it may have

immune modulatory properties. These data suggest that it may have therapeutic potential in cancer[103,107].

Many anti-inflammatory drugs such as non-steroid anti-inflammatory drugs, statins, and inhibitors of, for instance IL-1, IL-6, tumor necrosis factor and transforming growth factor beta, have been shown to have anti-neoplastic activity. Our findings emphasize that these and novel anti-inflammatory drugs should be further studied in controlled prospective trials[108]. Key questions relating to our findings are whether the inflammation was tumor-induced or if it was an unspecific systemic inflammation due to metastatic disease. And to what extent the inflammation is causing the poor outcome.

5.4 PAPER IV

The main findings in this paper were that enrichment of genes involved in inflammatory and immune response correlated with PFS to TT, whereas down-regulation of genes involved in proliferation and up-regulation of genes involved in immunological processes significantly correlated with longer PFS to ICI. Our results are in agreement to what has been presented in the literature, as described in detail in the discussion section of paper 4[51,52,109,110].

Our findings that a well-functioning immune system in the TME is important for achieving better results with TT make sense based on previous evidence that MAPK pathway activation causes immune evasion in melanoma cells. This is thought to occur through increased secretion of factors that inhibit immune response (i.e. IL-10, IL-6, IL-1) or impairing tumoral antigen presentation, and treatment with MAPKi may revert immune suppression of melanoma cells[8,111,112]. Based on these data, the triple combination of anti PD-1 or anti-PD-L1 plus BRAFi+MEKi, was compared to BRAFi+MEKi in clinical trials[40,113,114]. The data is not yet fully mature but may in the future support that the triplet is associated with somewhat higher activity, but also increased toxicity. It would be interesting to understand if there was a different degree of benefit from the triplet treatment in patients with low expression of immune genes and/or high expression of proliferation genes, whereas patients with a more immunocompetent TME and less proliferative tumors would not need the triplet?

We have also observed a down-regulation of immune genes (IFN gamma and alfa) in tumor samples collected at disease progression from two subjects treated with TT compared with matched pre-treatment samples, which could potentially explain the poor clinical outcome often noted when treating patients with progressive melanoma after MAPKi with ICI (cross-resistance)[115].

Finally, a body of evidence has suggested different strategies to turn “cold tumors” into “hot tumors” by combining chemotherapy, radiotherapy, kinase inhibitors, etc, with ICI based only on the tumors or the TME immune phenotype (immune-desert, immune-excluded or immune-inflamed) as recently reviewed by Olza et al[116]. Our study suggests that these combinations should be tested also in the context of expression of proliferative gene sets in melanoma.

5.5 LIMITATIONS AND STRENGTHS

The main limitation of our studies is that although the material was prospectively collected in a systematic way, the analysis was retrospective and performed in small cohorts of patients without control groups. Our results are, however, consistent with other reports in the literature. Moreover, we were able to identify some novel biologically plausible biomarker candidates. Finally, our studies emphasize the importance of systematically collecting clinical data together with biological material from real world patients in order to advance medicine.

6 CONCLUSIONS AND FUTURE PERSPECTIVES

6.1 PAPER I

EPHA2, MET, FLI1 and CD13/ANPEP over-expression are possible mechanisms of resistance to TT and should be further studied as predictive biomarkers. Studies combining drugs targeting these proteins with MAPKi in upfront or later during the course of metastatic CMM are warranted.

6.2 PAPER II

Increased levels of circulating EV miRNAs let-7g-5p and 497-5p, early during treatment with TT were identified as possible candidates as predictive biomarkers for response and PFS to TT. Additional studies further investigating these miRNAs and possible functional mechanisms behind these findings are recommended.

6.3 PAPER III

Increased relative levels of acute-phase inflammation proteins in plasma, and lower relative levels of plasma apolipoproteins possibly predict worse clinical outcome to TT and ICI. Larger controlled studies of these candidate proteins and their role in melanoma are motivated. Studies investigating combination of anti-inflammatory drugs with MAPKi and ICI should be considered.

6.4 PAPER IV

Upregulation of genes involved in cellular proliferation were identified as possible negative predictive biomarkers to ICI, whereas down-regulation of immune genes correlated with impaired effect of TT. These findings motivate the design of trials combining other treatment modalities with TT and ICI, at least in subset of patients with these identified characteristics.

Unraveling molecular mechanisms behind CMM development such as constitutive activation of MAPK pathway and tumor immune evasion by PD-1/PD-L1 interaction, has led to the discovery of new drugs which improve patient survival. However, primary and acquired resistance occur and only a small subset of patients has long-term benefits from these treatments. To overcome resistance and further improve clinical outcome, mechanisms behind resistance must be further explored and predictive biomarkers identified. CMM is a disease with high inter-tumoral and clonal heterogeneity making it difficult to find only one or few

biomarkers relevant for all patients. There are therefore many small subgroups of patients and even clones of tumor cells that demand different treatment approaches. Studying small subgroups is a challenge and requires a large number of patients in order to generate robust scientific results.

Additional important issues that must be taken into consideration more seriously, is global access to successful medicines and economic sustainability, which is threatened even in developed countries and can only be achieved by a rational use of these new therapies.

We have been aware of the importance of individualized treatment for decades and, although considerable advances have been made in this field, a lot of work remains to be done. Systematic global collaboration and data sharing, implementation of internationally standardized biobank sampling and analysis following established best practices routines and, harmonization of molecular data with clinical data are urgently needed[117].

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